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Ovarian Development and Sex Steroid Hormones during the Reproductive Cycle of *Rana esculenta* Complex

Alberta Maria Polzonetti-Magni, Roberta Curini[1], Oliana Carnevali, Claudio Novara, Massimo Zerani and Anna Gobbetti

Dipartimento di Biologia Cellulare, Università di Camerino, Via Camerini 1, 62032 Camerino, and [1]Dipartimento di Scienze Chimiche, Università di Camerino, Via S. Agostino 1, 62032 Camerino, Italy.

ABSTRACT—The aim of this work is to investigate the ovarian development of *Rana esculenta* living in Colfiorito pond (820 m a. s.l.). This process depends on a complex interplay between the estradiol-induced hepatic vitellogenin and the uptake of vitellogenin by the ovary. In order to establish whether the ovarian growth may depend on hydratation process as well, the ovaries have been analyzed—during the annual cycle—by thermo-analysis. As far as we know, this method has been employed for the first time to lower vertebrate tissues with the present work. Vitellogenin titre varies according to a temporal pattern which is characteristic of this mountain population and is well correlated with plasma estradiol concentration. Samples of ovarian tissues, monitored by thermal analysis, show that, in the pre-spawning period three different kinds of water are bonded to the tissues with different energies of interaction. In the ovulatory period, the water is released in one process only. Samples of the post-ovulatory period give the curves, which can be plotted in-between the pre-spawning and ovulatory ones. In conclusion, the present results describe how two different kinds of water are released during the ovarian development. The low weight ovaries, with small oocytes, show a high water percentage which is lost in only one step; the high weight ovaries show a low water percentage, that is lost in three steps. Therefore, in the recovery phase, the ovarian growth, seems mainly due to yolk storage and only partly to the hydratation process which, moreover, is closely related with plasma sex steroid concentration.

INTRODUCTION

The ovarian development in anuran amphibian *Rana esculenta* is cyclical: the growth starts in September (recovery phase), stops temporarily during winter stasis and resumes just before ovulation (April-May). This development is regulated by several environmental cues (temperature and photoperiod) and endocrine mechanisms.

Previous studies [1] have shown that the length of the recovery phase depends mainly on temperature. In fact the recovery phase in the population of green frogs living in the mountains is shorter than that in the population living at sea level. Such a difference depends on an early drop of mountain temperature in autumn. The ovarian growth is essentially due to the storage of vitellogenin, synthesized by the liver and then released in the blood. Moreover, also hydratation process—not yet well known—plays a part in ovary weight increase [2].

The vitellogenesis occurs under hormonal control: in fact, as in other amphibian species [3–5], also in *Rana esculenta* [6, 7], estradiol is the hormone most responsible for the vitellogenin synthesis, while the gonadotropins support the ovarian uptake. Nevertheless, Gobbetti *et al.* [8] pointed out a significant intervention of gonadotropins in inducing hepatic vitellogenin synthesis. It is certain, however, which is the role of hydratation in ovarian growth and how it is regulated by sex steroid hormones. Therefore it seemed a good opportunity to investigate these aspects by thermal

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analysis; as far as we know, this was the first application to lower vertebrate tissues. At the same time, the plasma vitellogenin and sex hormore titre were monitored during the reproductive cycle of *Rana esculenta*. All samples were taken in the field to avoid the stress of captivity on plasma steroid concentration [9].

MATERIALS AND METHODS

Animals and tissues

Ten female frogs, *Rana esculenta* complex, were monthly captured in the moutain poud of Colfiorito (820 m above sea level) and anesthetized with MS 222 (Sandoz). Previous data indicate that MS 222 does not affect the hormone profiles in *Rana esculenta* [10, 11]. Blood was collected into heparinized centrifuge tubes by a heparinized glass capillary which was inserted into the *conus arteriosus*. After centrifugation, individual plasma samples were stored at $-20^{\circ}C$ until hormonal determinations. Ovaries-taken from these animals- were removed and weighed. One of these was kept at $-20^{\circ}C$ until thermal analysis and the other one was placed in amphibian Krebbs-Ringer solution. The latter was opened carefully and dissected in order to separate follicular oocytes, as previously described [1]. The dissected oocytes were screened by granulometric sieves into three classes: black atretic follicles (ø: 0.4–0.69 mm), previtellogenic follicles (ø: 0.70–1.2 mm), and vitellogenic follicles (ø: 1.21–2.0 mm).

Thermal analysis

The water interactions, in the biological systems, are in function of the hydrogen bonds, of the Van der Waals forces and the London forces etc.; so the water in the biolobical systems is bound to the matrix by different energies, also in relation to the different kinds of molecules present in the matrix. When a biological system is heated, the water is released in different successive steps in function of the necessary activation energy to be obtained in the break of each different bond from its water-matrix. Each step will thus represent a particular onertype of water characterized by one particular value of the interaction energy with the matrix.

The different kinds of water and their percentage were determined by thermal analysi (TG) as described by Wendlandt [12], using a Perkin Elmer TG-S2 thermobalance equipped with a data station. The operational atmosphere was air or oxygen at a flow of 150 ml min^{-1}. An inert atmosphere was also used in the preliminary phase to check whether oxidation phenomena may anticipate the decomposition of the organic matter which would interfere with the water percentage determination. The temperature program chosen was $10^{\circ}C\ min^{-1}$ and the sample weight ranged between 20 and 50 mg.

Vitellogenin assay

Male frogs were estrogenized with estradiol silastic tubes implanted into the ventral cavity and were kept in water tanks on a diet of mealworms. After 20 days, the estrogenized males were anaesthetized on MS 222 and blood samples were collected as previously described. Vitellogenin was purified from estrogenized serum by the EDTA-$MgCl_2$ method [13] and then chromatographized on DEAE cellulose (Whatman).

Using a protocol developed in our laboratory, purified vitellogenin has been used to raise antibodies. When antiserum was tested by immunoblotting, strong reactivity was observed vs. isolated vitellogenin. Control experiments were made with preimmune serum. The antibodies recognized a protein present in estrogenized frog serum but failed to react against control serum. The data lead us to conclude that i) the antiserum is specific for vitellogenin and ii) the antigen was purified to a high extent. So the α-VTG Abs were used to determine the plasma vitellogenin titre by enzyme-linked assay (ELISA). The serum of ten female frog, used as antigen, was absorbed on polystyrene microplates. Rabbit vitellogenin antiserum was diluted 1 : 1000 and was incubated for 2 hr at room temperature. The conjugate was alkaline-phosphatase goat antirabbit immunoglobulin, diluted 1/1000 and incubated for 2 hr at room temperature. P-nitropheyl-phosphate was employed as enzyme substrate for 60 min, the results were expressed in absorbance units at 405 nm. The calibration curve shown in Figure 1 enables

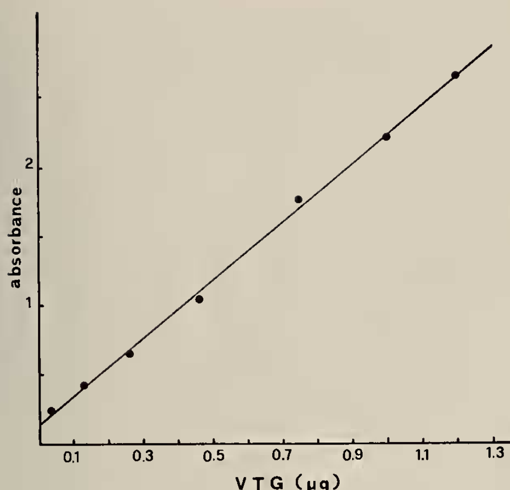

FIG. 1. Calibration curve of *Rana esculenta* plasma vitellogenin as determined by enzyme-linked immunosorbent assay. The absorbance was plotted versus the relative amount of antigen placed in the hole. Each point represents the mean of three independent measurements.

the antigen titre to be measured in all the sera collected.

The sensitivity of the ELISA method was 3 ng (intraassay 6%; interassay 11%).

Protein concentration was determined by the procedure which uses bovine serum albumin as standard.

Plasma hormone determinations

Plasma samples, taken monthly from ten females, were extracted with ether; subsequently radioimmunological analyses (RIA) of estradiol-17β (E), progesterone (P) and androgens (A), were carried out as previously described [15].

The following sensitivities were observed: testosterone 5 pg (intraassay, 7%; interassay, 13%; progesterone, 7 pg (intraassay, 4%; interassay, 8%). Testosterone was not separated from dihydrotestosterone and therefore, since its antibody reacts with dihydrotestosterone, the data are expressed as "androgen". The antisera were provided by Dr G. Bolelli, Centro di Fisiopatologia della Riproduzione, C. N. R., Bologna.

All numerical data of the present experiments were analyzed using an ANOVA method.

RESULTS

The ovarian growth, during the reproductive period, has been evaluated considering the oocytes diameter. During the ovulatory period (May), the ovary comprises mainly full-growth oocytes (1.21–2.0 mm). In the post-reproductive period (July), the number of full-growth oocytes decreases and the smallest oocytes look atretic as in the previtellogenic stage. During the recovery phase (September), the number of oocytes entering the vitellogenic cycle increases and the cells continue their growth and gradually accumulate in the later stages.

The plasma vitellogenin profile is correlated with ovarian composition. The ovary weight presents its main peak in winter (stasis period), before ovulation (April). In the post-reproductive period, because the oocytes are ovulated, the ovary weight is very low, and begins to grow during the recovely phase (autumn), to reach its maximum the next winter, as can been seen in Figure 2.

These modifications, when submitted to analysis of variance, were statistically significant ($p<0.001$).

Also the variations of plasma vitellogenin titre were statistically significant ($p<0.001$). The high vitellogenin levels both in stasis and in the recovery period are correlated with the ovary weight. On the contrary, in the reproductive and post-reproductive period, the ovary weight is low because the ovulation and vitellogenin accumulates in the plasma. The estradiol profile agrees with our previous dat [15]. The significant increase ($p<0.001$) of plasma estradiol levels in July is responsible for the induction of hepatic vitellogeinin synthesis which in turn allows recovery of the ovarian weight [8].

Samples of ovarian tissues, corresponding to the different periods of the ovarian cycle, were analysed by TG to assay the hydratation process. The curves obtained show three types of behavior, each characteristic of a specific period of the cycle. Figure 3 shows a characteristic trend of water percentage in the fullgrowth oocytes (pre-reproductive period). In fact the water is released through three different processes occurring within

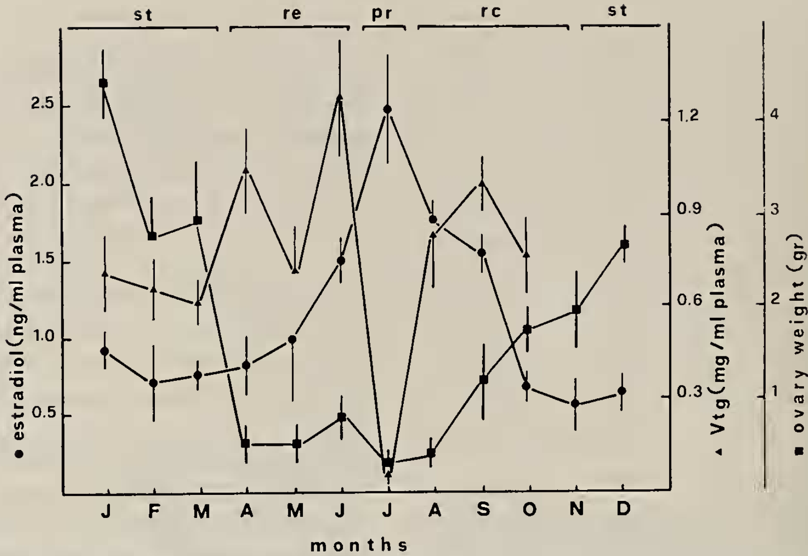

Fig. 2. Time course of vitellogenin and estradiol titre in the plasma of *Rana esculenta* cycle includes four periods: repreductive period (re), post-reproductive (pr), recovery (rc) and stasis (st). Number of frogs used is shown in the text.

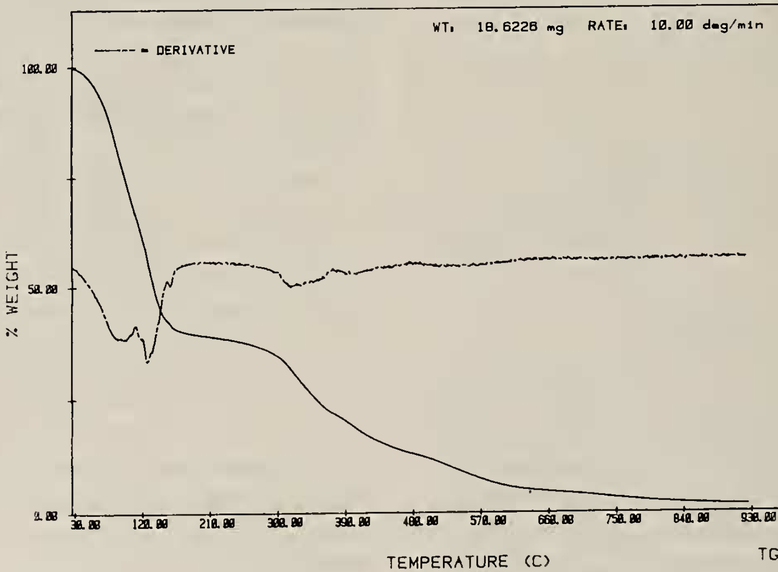

Fig. 3. Thermogravimetric analysis (TG) of *Rana esculenta* ovaries during the pre-ovulatory period.

the temperature intervals of 25–105°C (1st process), 105–155°C (2nd process) and 155–210°C (3rd process). They ssuggest that three different kinds of water are bonded to the tissues with different energies of interaction.

The thermal behavior is surprisingly constant during a very long interval of time, especially in consideration of the fact that such samples were obtained from 10 different animals. The percentage of total water contained in the examined tissues ranged between 45 and 55%. The curves corresponding to the ovulatory period samples (low weight ovaries) show that the water is lost in one process only, occurring in the temperature

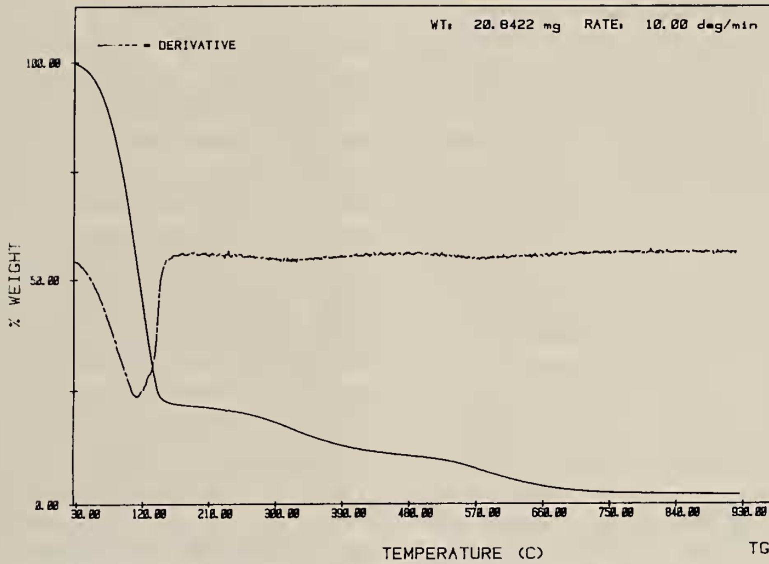

FIG. 4. Thermogravimetric analysis (TG) of *Rana esculenta* ovaries during the ovulatory period.

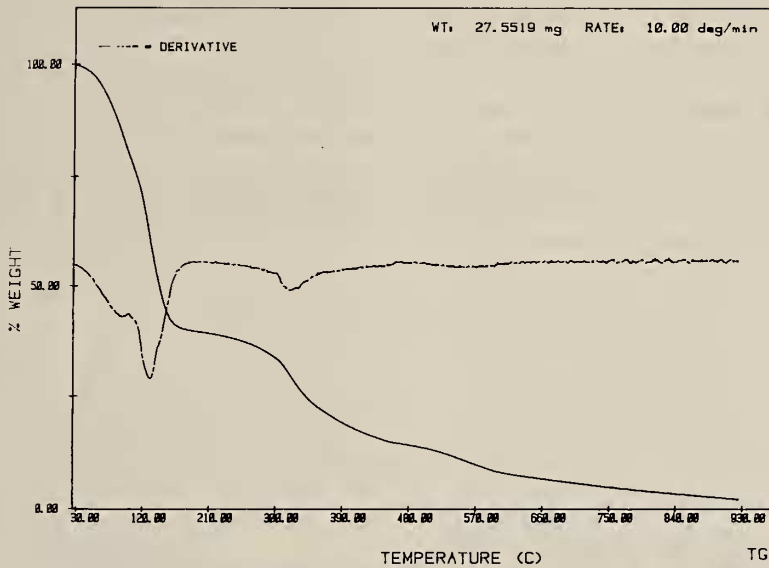

FIG. 5. Thermogravimetric analysis (TG) of *Rana esculenta* ovaries during the post-reproduction period.

interval of 25–180°C with a functuation as described above. The thermal behavior is quite constant and only rarely is the peak split in two different tips. The percentage of total water contained in the examined tissues ranged between 65 and 75% (Fig. 4). Samples of the post-ovulatory period originate TG curves, that are plotted in between those for the ovulatory and pre-spawning period. Figure 5 depicts an example of typical intermediate TG behavior.

DISCUSSION

Ovarian development in non-mammalian vertebrates entails a complex interplay between the ovary and various other organs [16]. A high

molecular weigh yolk precursor (vitellogenin) is synthesized and secreted by the liver and subsequently transferred to the ovary by endocytic uptake. Since synthesis and uptake of vitellogenin are both hormone-dependent, the turn-over of vitellogenin in the blood appears to be a function of the hormonal balance influencing both processes. In *Rana esculenta,* Gobbetti *et al.* [17] found that estradiol-17β induces vitellogenin synthesis, as in other amphibian species. Recent results also suggest that the availability of specific liver receptors is strongly related to vitellogenin hormonal regulation [18].

The present data show a strict relationship between vitellogenin titre and ovarian behavior as witnessed by ovary weight during stasis and the recovery phase. On the contrary, when the ovary weight is very low in May (full-growth oocytes are ovulated), high concentrations of vitellogenin accumulate in the plasma.

The results obtained on ovarian tissue checked by TG at the different periods of the cycle support the hypothesis that the determination of water content could be important in the explanation of ovarian growth. In fact, our results indicate that two different kinds of water are released during ovarian development. The low weight ovaries with small oocytes show a high water parcentage, which is lost in only one step. In contrast, high weight ovaries (full-growth oocytes) show a low water percentage, that is lost in three steps. In the high weight ovaries, therefore, the water percentage is lower than in small ovaries. The water of the full growth oocytes, released at high temperature (210°C), is probably connected with yolk macromolecules. On the contrary, the high water percentage of small oocytes is released at low temperature. Therefore, the increase of ovarian weight during the recovery period of *Rana esculenta* seems mainly due to the storage of yolk and only partly to hydratation processes [19].

The water percentage is well related with the plasmatic sex steroid concentrations. In *Rana esculenta* the main peak of plasma androgen levels was found in the reproductive period [20]. Also in these experiments the androgen titre is higher when the water percentage is about 50% (Fig. 6). During the post-reproductive period the high titre of plasmatic estradiol agrees with the high percentage of water in low weight ovaries. It is already known that steroid hormones in fish and in mammals are involved in tissues hydratation process [21, 22]; while, as far as amphibians are concerned, other hormones, such as prolactin in urodeles, may play an important role. It is therefore worthwhile studying more in depth in anurans the implication

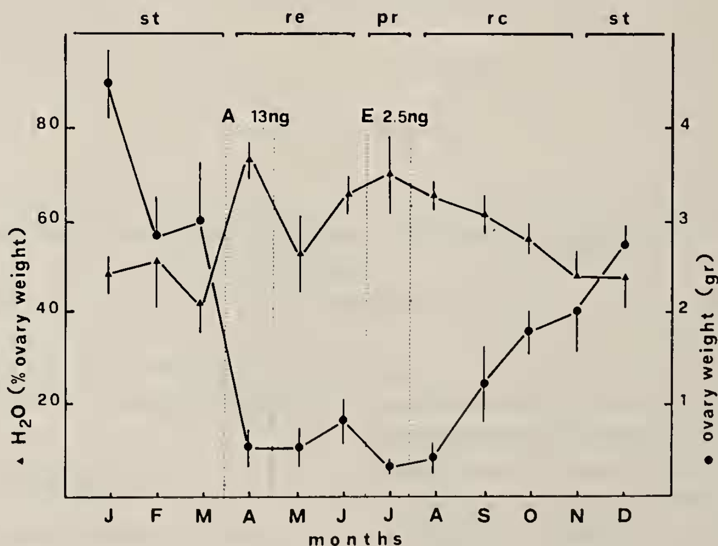

Fig. 6. Relationship between ovary weight, ovary water percentage and plasma sex steroid levels (A = androgens; E = estradiol-17β), in the different phases of female *Rana esculenta* reproductive cycle.

of other hormones in the hydratation process.

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REFERENCES

1 Polzonetti-Magni, A., Pagano, M., Gobbetti, A., Bellini-Cardellini, L. and Botte, V. (1984) Accrescimento annuale del-l'ovario in popolazioni di *Rana esculenta,* viventi in localitá con differente andamento climatico. Riv. Biol., **77**: 97–103.

2 Pierantoni, R., Varriale, B., Simeoli, C., Di Matteo, L., Milone, M., Rastogi, R. K. and Chieffi, G. (1983) Fat body and autumn recrudescence of the ovary in *Rana esculenta.* Comp. Biochem. Physiol., **76A**: 31–35.

3 Wallace, R. A. and Jared, D. W. (1969) Studies on amphibian yolk. VIII. The estrogen-induced hepatic synthesis of a serum lipophosphoprotein and its selective uptake by the ovary and transformation into yolk platelet proteins in *Xenopus laevis.* Dev. Biol., **19**: 498–526.

4 Wallace, R. A. (1978) Oocyte growth: non mammalian vertebrates. In "Evolution of the Vertebrate Ovary". Ed. by R. E. Jones, Plenum Press, New York, pp. 469–502.

5 Searle, P. F. and Tata, J. R. (1981) Vitellogenin gene expression in male *Xenopus* hepatocytes during primary and secondary stimulation with estrogen in cell cultures. Cell, **23**: 741–746.

6 Giorgi, F., Gobbetti, A. and Polzonetti-Magni, A. (1982) Variations in the vitellogenin titre during the reproductive cycle of *Rana esculenta* L. Comp. Biochem. Physiol., **72B**: 501–506.

7 Varriale, B., Pierantoni, R., Di Matteo, L., Minucci, S., Milone, M. and Chieffi, G. (1988) Relationship between estradiol-17β seasonal profile and annual vitellogenin content of liver, fat body, plasma, and ovary in the frog (*Rana esculenta*). Gen. Comp. Endocrinol., **69**: 328–334.

8 Gobbetti, A., Polzonetti-Magni, A., Zerani, M., Carnevali, O. and Botte, V. (1985) Vitellogenin hormonal control in the green frog, *Rana esculenta.* Interplay between estradiol and pituitary hormones. Comp. Biochem. Physiol., **82A**: 855–858.

9 Licht, P., Mc Creery, B. R., Barnes, R. and Pang, R. (1983) Seasonal and stress related changes in plasma gonadotropins, sex steroids and corticosterone in the bullfrog, *Rana catesbeiana.* Gen. Comp. Endocrinol., **50**: 124–145.

10 D'Istria, M., Delrio, G., Botte, V. and Chieffi, G. (1974) Radioimmunoassay of testosterone, 17β-oestradiol and oestrone in the male and female plasma of *Rana esculenta* during sexual cycle. Steroids Lipids Res., **5**: 42–48.

11 Pierantoni, R., Iela, L., Delrio, G. and Rastogi, R. K. (1984) Seasonal plasma sex steroid levels in the female *Rana esculenta.* Gen. Comp. Endocrinol., **53**: 126–134.

12 Wendlandt, W. W. (1986) Thermal analysis. Anal. Chem., **58**: 1R–6R.

13 Wiley, H. S. and Dumont, J. N. (1978) Stimulation of vitellogenin uptake in stage. IV. *Xenopus* oocytes by treatment with chorionic gonadotropin *in vivo.* Bio. Reprod., **18**: 762–771.

14 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem., **193**: 265–275.

15 Polzonetti-Magni, A., Botte, V., Bellini-Cardellini, L., Gobbetti, A., Crasto, A. (1984) Plasma sex hormones and post-reproductive period in the green frog, *Rana esculenta* complex. Gen. Comp. Endocrinol., **54**: 372–377.

16 Follett, B. K., Redshaw, M. R. (1974) The physiology of vitellogenesis. In "Physiology of Amphibia". vol. 2, Ed. by B. Lofts, Academic Press, New York, pp. 219–308.

17 Gobbetti, A., Polzonetti-Magni, A., Zrani, M. and Paolucci, M. (1983) Vitellogenin synthesis induced by estradiol-17β in the green frog, *Rana esculenta* complex. Rend. Acc. Sci. Fis. Mat., **4**: 60.

18 Paolucci, M. and Botte, V. (1988) Estradiol-binding molecules in the hepatocytes of the female water frog, *Rana esculenta,* and plasma estradiol and vitellogenin levels during the reproductive cycle. Gen. Comp. Endocrinol., **70**: 466–476.

19 Rastogi, R. K., Izzo-Vitiello, I., Di Meglio, M., Di Matteo, L., Franzese, R., Di Costanzo, M. G., Minucci, S., Iela, L. and Chieffi, G. (1983) Ovarian activity and reproduction in the frog, *Rana esculenta.* J. Zool. Lond., **200**: 233–247.

20 Lupo, C., Zerani, M., Carnevali, O., Gobbetti, A. and Polzonetti-Magni, A. (1988) Testosterone binding protein in the encephalon and plasma sex hormones during the annual cycle in *Rana esculenta* complex (Amphibia Ranidae). Monitore Zool. Ital. (N. S.), **22**: 133–144.

21 Babiker, M. M. and Ibrahim, H. (1979) Studies on the biology of reproduction in the cichlid *Tilapia nilotica* (L.): effects of steroid and trophic hormones on ovulation and ovarian hydration. J. Fish Biol., **15**: 21–30.

22 Warren, J. C. and Crist, R. D. (1973) Effects of ovarian steroids on uterine metabolism. In "Handbook of Physiology", Ed. by R. O. Greep and E. B. Aswood, publ. pp. 49–67.